JOURNAL OF BACTERIOLOGY, July 1986, p. 160–167
0021-9193/86/070160-08$02.00/0

Vol. 167, No. 1

Effects of Signal Sequence Mutations on the Kinetics of Alkaline Phosphatase Export to the Periplasm in *Escherichia coli*

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Received 10 January 1986/Accepted 22 April 1986

We isolated a collection of mutants defective in the export of alkaline phosphatase to the periplasm. Two classes of mutants were obtained: one class with lesions unlinked to the *phoA* gene and a second class harboring linked mutations. Among the former class, one mutant is cold sensitive for growth and may be defective in a component of the *Escherichia coli* secretory apparatus. Included in the latter class are 47 mutants which are characterized in detail in this report. To facilitate DNA sequence analysis of these mutants, we devised a convenient method that relies on homologous recombination in vivo to transfer *phoA* mutations from the bacterial chromosome directly onto the genome of a single-stranded M13 phage vector. DNA sequence analysis revealed that our collection of mutants comprises six unique mutations, all of which reside in the *phoA* signal sequence coding region and lend further support to the notion that the length of the hydrophobic core of the signal sequence is crucial for its function in protein export. Kinetic studies showed that in these mutants, the small fraction of alkaline phosphatase which succeeds in reaching a periplasmic location, despite a defective signal sequence, is translocated across the membrane in a slow, posttranslational fashion.

All periplasmic and outer membrane proteins in *Escherichia coli* are synthesized initially in precursor form with an amino-terminal signal sequence that is proteolytically removed during or after export. Analysis of mutants has shown that a functional signal sequence is required for export; in several cases, proteins whose signal sequences have been mutationally altered cannot be exported, and instead, the precursor form of those proteins remains in the cytoplasm (3, 27). However, the specific step in export which is affected by signal sequence mutations has not been determined. It has been variously proposed that the signal peptide inserts the nascent protein into the membrane (19), binds to a cytoplasmic secretion complex (analogous to the eucaryotic signal recognition particle) (13, 25), or functions at both steps (3).

We would like to better understand how the structure of the signal peptide determines its function. From numerous genetic studies (1, 3), a general scheme for the isolation of mutants with defects in secretion has been developed. In this scheme, strains are used that contain gene fusions in which the amino-terminal region of β-galactosidase (encoded by the *lacZ* gene) is replaced by the amino-terminal portion of an exported protein, including its signal sequence. In most cases the hybrid protein produced by such strains is localized to the cytoplasmic membrane. This type of membrane-bound hybrid protein often shows abnormally low β-galactosidase enzymatic activity, so that, as a result, strains bearing such fusions can exhibit a Lac^- phenotype. This is the case with β-galactosidase fusions to the periplasmic proteins alkaline phosphatase (AP) (28) and maltose-binding protein (33) and to the outer membrane protein LamB (14). The low enzymatic activity of the hybrid proteins appears to be due to their membrane location, since mutations (e.g., in the signal sequence) which cause the hybrid proteins to be localized in the cytoplasm instead of the inner membrane restore a Lac^+ phenotype. Selection for Lac^+ derivatives with these strains demands that at least a fraction of the hybrid protein be internalized and therefore provides a direct means for obtaining mutants with defects in secretion. This approach has yielded mutations both in the signal sequences of several proteins and in certain other bacterial genes whose products appear to play an important role in protein export (24, 29, 33).

Using this selection scheme, we previously reported the isolation of two mutants with alterations in the signal sequence of the periplasmic enzyme AP (29). AP, encoded by the *phoA* gene, is a dimer composed of identical monomeric subunits of a molecular weight of 49,700 (6). For it to be enzymatically active, several steps are required including dimerization, the formation of intrachain disulfide bonds, and the incorporation of zinc ion (37). We found that the cytoplasmic precursor AP found in strains bearing *phoA* signal sequence mutations was not enzymatically active. One hypothesis to account for this absence of activity is that the internalized precursor may be unable to form the necessary disulfide bonds in the cytoplasm where there is a high reducing potential. Both signal sequence mutants isolated previously retained a low residual amount of AP activity that could be accounted for by the inefficient export of a small fraction of mutant AP product into the periplasm. Thus, the amount of AP enzymatic activity detected in any mutant provides a precise measure of the extent of AP export in that particular strain. Because of this feature, the AP system can be used to accurately quantitate the effects of signal sequence mutations.

Since AP offers this advantage, we sought to isolate additional secretion-defective mutants by using a *phoA-lacZ* fusion. In this paper, we report the isolation of a large collection of mutants impaired for export of the hybrid protein and characterize the effect of these mutations on secretion of AP. These studies yielded both new signal sequence mutations as well as new mutations unlinked to the *phoA* gene. All of the signal sequence mutants show some residual AP export. Analysis of the kinetics of this export,

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain or bacteriophage	Genotype or bacterial genes carried	Origin
Strain		
MC1000	F⁻ *araD139* Δ(*ara-leu*)*7697* Δ*lac74 galE galK rpsL*	Beckwith collection
MPh42	MC1000 *phoR*	Beckwith collection
MPh1061	MPh42 *phoA61*	Michaelis et al. (29)
MPh1068	MPh42 *phoA68*	Michaelis et al. (29)
MPh1021	MPh42 *phoA21*	This study
MPh1073	MPh42 *phoA73*	This study
MPh1082	MPh42 *phoA82*	This study
MPh1093	MPh42 *phoA93*	This study
MPh44	MC1000 Δ(*phoA-proC*) *phoR tsx*::Tn*5*	Beckwith collection
SM655	MPh44 F′ *lac*	Michaelis et al. (29)
SM656	SM655 *prlA3 rpsE*	Michaelis et al. (29)
Bacteriophage		
λ456	Φ(*phoA-lacZ*)*456*(Hyb) *lacYA′*	Michaelis et al. (29)
f1 R234		Inouye et al. (17)
f1 SM102	*phoA*⁺	This study
f1 SM103	*phoA* Δ*Pvu*II	This study
f1 *phoA* Δ*Pvu*II	*phoA* Δ*Pvu*II	Michaelis et al. (29)
Plasmid pHI-1	*phoA*⁺	Inouye et al. (17)

taken together with results from other laboratories (9, 20–22, 35), indicates that there exists a posttranslational mode of protein export in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, media, and chemicals. The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1. Media for growth and plating of bacteria, including TYE, LB, and M63, have been described previously (30). For reasons we do not understand, MPh42 and its derivatives grew poorly in M63 medium containing 0.4% glucose and supplemented simply with thiamine (1 μg/ml) and leucine (40 μg/ml). To obtain efficient growth of these strains, we supplemented the medium with a 1:100 dilution of a mixture of 19 amino acids (lacking methionine) at 5 mM each.

To distinguish slight differences in AP activity among strains which produce extremely low amounts of the enzyme, colonies were streaked on Tris high-P_i medium (38) containing 40 μg of 5-bromo-4-chloro-3-indolylphosphate (XP) (Bachem Co.) per ml, which is a substrate for AP. *p*-Nitrophenyl phosphate used in the AP enzyme assay was purchased from Sigma Chemical Co., St. Louis, Mo.

Genetic procedures. Lac⁺ derivatives of MPh42(λ456) were selected essentially as described previously (29). Briefly, a 0.1-ml sample from each of 100 independently grown, saturated cultures was plated on MacConkey-lactose agar. After incubation for 2 days at 42°C, red papillae appeared and continued to increase in number for several days. To prevent biasing the selection for a particular class of mutants, an effort was made to choose papillae that appeared at different times over a period of 2 to 6 days. One hundred independent mutants were purified on MacConkey-lactose agar at 42°C and screened for the ability to grow on the same medium at 30°C.

Cured, nonlysogenic derivatives of the Lac⁺ mutants were obtained after UV irradiation as reported previously (29). Lysogens of λ456 and its mutant derivatives were made as previously described (29).

AP assay. Cultures were grown at 37°C to the stationary phase in LB or M63 medium containing glucose and amino acids as indicated above. Cells were pelleted, washed twice to remove all traces of inorganic phosphate, and assayed as described previously (7), except that cells were permeabilized with 5% chloroform and 0.0025% sodium dodecyl sulfate before the addition of *p*-nitrophenyl phosphate. Units are calculated as previously reported (7).

Preparation of DNA and DNA sequencing. High-titered lysates of f1 phage derivatives which harbored *phoA* mutations and single-stranded DNA to be used as a template for DNA sequencing were prepared as previously reported (29). DNA sequencing by the dideoxy chain termination method was performed essentially as described previously (29), using a sequencing kit purchased from Pharmacia P-L Biochemicals. The 190-base-pair *Hin*dIII-*Pvu*II restriction fragment used as a source of single-strand primer was kindly given to us by Hiroshi Inouye.

[^{35}S]methionine labeling, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cultures were grown at 30°C to the early exponential phase in M63 minimal medium containing the supplements indicated above. Cells were labeled with 20 μCi of [^{35}S]methionine per ml (approximately 1,200 Ci/mmol) for 1 min at 30°C. Incorporated radioactivity was chased for various lengths of time by the addition of unlabeled methionine at a final concentration of 0.05%. The chase was terminated by rapidly chilling samples on ice. Labeled cultures were subjected to immunoprecipitation as previously described (28), using antiserum against periodate-treated AP (provided by Jeff Garwin). Immunoprecipitates were electrophoresed and autoradiographed as described previously (28). We used gels that were 28 cm in length and contained 10% acrylamide and 0.26% bisacrylamide.

DNA techniques. Restriction enzyme digestion, filling-in reactions with DNA polymerase I (Klenow fragment), ligation, transfection, and transformation were all performed by published techniques (26) or as suggested by the manufacturer. Enzymes were obtained from standard sources.

Construction of the single-stranded M13 phage, f1 SM103. f1 SM103 is a single-stranded bacteriophage whose double-stranded replicative-form (RF) DNA is suitable as a target for recombining *phoA* signal sequence mutations so they can be directly sequenced by the dideoxy DNA-sequencing technique. The vector consists of phage f1, an M13 varient (17), which harbors an insert containing a partially deleted *phoA* gene (see Fig. 2). f1 SM103 was constructed in two steps via a *phoA*⁺-bearing intermediate phase, f1 SM102.

To construct f1 SM102, DNA from plasmid pHI-1, which carries the *phoA*⁺ gene on a 4-kilobase *Mlu*I-*Xho*I restriction fragment, was digested with *Xho*I and *Mlu*I. The cohesive ends were made flush with DNA polymerase I (Klenow fragment), and the resulting blunt-ended fragments were ligated to f1 234 RF DNA which had been digested with *Hin*dIII and then filled in. To identify recombinant phage bearing the *phoA* gene, we screened for blue plaques on XP-containing media upon transfection of the ligation mix into the Δ*phoA* strain SM655. Several phage which produced blue plaques were purified.

One such phage, f1 SM102, was used in the subsequent construction since it contained the *Mlu*I-*Xho*I fragment in the appropriate orientation to facilitate DNA sequence analysis of the *phoA* signal sequence, using a particular *Hin*dIII-*Pvu*II restriction fragment as a primer (see Fig. 2). f1 SM102

contains two *Pvu*II sites whose positions, relative to the *phoA* gene, were known from previous studies (18). The *Pvu*II-*Pvu*II region includes the promotor and first half of the *phoA* gene so that deletion of this restriction fragment results in a nonfunctional gene. After digestion of f1 SM102 RF DNA with *Pvu*II, ligation, and transfection, white plaques were obtained on plates containing XP. The presence of the expected *Pvu*II deletion in one such phage was confirmed by restriction enzyme digestion. This phage was designated f1 SM103 (see Fig. 2).

Recombination of chromosomal *phoA* signal sequence mutations onto f1 SM103. To propagate phage f1 SM103 on mutant strains, 10^8 PFU of phage were added to a 5-ml log-phase culture, and the persistently infected culture was grown for 4 h at 37°C. Cells were removed by centrifugation, and the supernatant, which contained 10^{11} to 10^{12} PFU/ml, was heated for 15 min at 65°C to kill any remaining bacteria. To examine their PhoA phenotype, we plated phage with a *phoA* indicator strain, SM655 or SM656, on TYE agar containing 40 μg of XP per ml. The parental phage, f1 SM103, produced white plaques, while phage which incorporated the wild-type or mutant *phoA* alleles (all of which permit production of some residual AP activity) formed blue plaques. To detect blue plaques, approximately 30,000 PFU of phage which had been propagated on a mutant strain were plated with indicator cells in a soft-agar lawn on a single petri plate. Typically one or two blue plaques were found on each plate. Phage from such plaques were purified three times and used for DNA sequence analysis.

RESULTS

Isolation of mutants impaired in localization of a *phoA-lacZ*-encoded hybrid protein. We sought to obtain a large collection of mutants defective in the export of AP to the periplasm. We were interested both in mutations in the *phoA* gene itself and in mutations unlinked to *phoA* which might define new genes encoding components of the procaryotic secretory apparatus. Two *phoA* signal sequence mutants had been obtained previously by the selection procedure described above, which demands internalization of the hybrid protein encoded by the gene fusion *phoA-lacZ456* (29). To obtain new classes of mutants, we employed this same selection on a large scale and isolated 100 independent Lac$^+$ mutants.

As a first step in characterization, we determined whether the mutations responsible for the Lac$^+$ phenotype were genetically linked to the *phoA-lacZ* fusion. Since the fusion resides on a λ prophage integrated in the chromosome, we made a phage lysate from each mutant by UV irradiation and determined which lysates could transduce the Δ*lacZ* strain MPh42 to Lac$^+$. When tested in this way, 74 of the mutants yielded Lac$^+$ transducing lysates and thus contained mutations linked to the *phoA-lacZ* fusion. Further characterization of these mutants is described below.

In contrast, 26 of the mutants appeared to contain unlinked mutations, since they produced lysates which failed to give transduction to Lac$^+$. When screened for a conditional lethal phenotype, one of these strains was shown to be cold sensitive for growth. Moreover, this mutant exhibited an export defect, since it accumulated AP precursor (data not shown). The mutation in this strain which renders cells both cold sensitive and export defective appears to define a new essential gene, *secD*, encoding a component of the *E. coli* export machinery. A detailed description of *secD* will be reported elsewhere (C. Gardel, J. Hunt, S. Michaelis, and J. Beckwith, manuscript in preparation). The 25 remaining unlinked Lac$^+$ mutants were examined for an AP export defect, but no accumulation of precursor could be detected, and these mutants were not further studied.

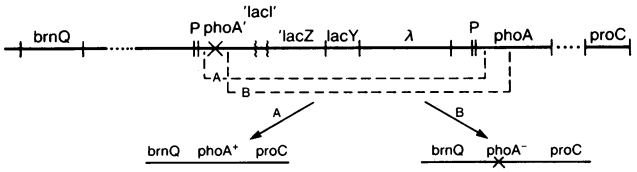

FIG. 1. Arrangement of chromosomal and phage genes in the lysogen MPh42(λ456). The phage λ456, which has no *att* site, was integrated by homologous recombination into the chromosome in strain MPh42. In the resulting lysogen, the λ prophage is located between the *phoA-lacZ* fusion and a directly repeated wild-type *phoA* gene. Here the fusion gene is shown containing a mutation. Excision of λ456 requires a recombination event between homologous *phoA* sequences as depicted by the dashed lines A and B. The precise site of crossover is random. Depending on which side of the mutation the crossover occurs, either a wild-type (A) or a mutant (B) *phoA* allele will be left behind on the chromosome. *brnQ* and *proC* are nearby genes.

Recombining linked mutations from the fusion gene onto the chromosomal *phoA* gene. It seemed likely that many of the 74 mutations linked to the *phoA-lacZ* fusion would reside in the *phoA* portion of the fusion, since it is this segment which provides export signals for the hybrid protein. To evaluate their effect on AP export, it was first necessary to transfer the mutations from the fusion to an intact *phoA* gene. Because it was integrated into the chromosome by homologous recombination, the *phoA-lacZ* fusion-bearing phage, λ456, is located between directly repeated *phoA* sequences in the MPh42(λ456) lysogen. Excision of λ456 can occur by a crossover event between these flanking sequences (Fig. 1), leaving behind on the chromosome a single intact *phoA* gene, with all phage and *lacZ* sequences removed. Because the fusions examined here bear a mutation, such a cured nonlysogenic derivative can contain either a wild-type or a mutant *phoA* gene, depending on the precise site of the crossover event. For cured derivatives in which the mutation has been transferred to the chromosomal *phoA* gene, relysogenization with λ456 will yield Lac$^+$ lysogens, whereas for cures carrying the wild-type *phoA* gene, no Lac$^+$ lysogens will be formed.

After excision of the phage, eight cured derivatives were obtained from each of the mutants containing lesions linked to the fusion. Of the 74 mutants tested, 47 yielded at least one cured derivative which produced Lac$^+$ colonies when relysogenized with λ456, indicating that the original mutation had been transferred from the fusion to the chromosomal *phoA* gene. For the remaining 27 mutants, none of the eight cured derivatives had the potential to regenerate Lac$^+$ lysogens with λ456. Even isolation of a second set of eight nonlysogenic derivatives produced no cures retaining the mutation. Two different classes of mutations in the fusion gene can be envisioned that could result in internalization of β-galactosidase activity but would be difficult or impossible to transfer to the chromosome by homologous recombination: (i) mutations that would result in a lethal species of AP when present in an intact *phoA* gene, and (ii) mutations whose position at or near the fusion joint would preclude their frequent recombinational transfer to the chromosomal *phoA* gene. Among this second class could be mutations far away from the *phoA* signal sequence and near the middle of the gene which interfere with export. Mutations which

TABLE 2. Distribution of mutants defective in export of AP

Mutant class	AP activity[a] (%)	No. of mutants	Residue altered by mutation[b]	Allele designation[c]
1	30 (27–30)	2	Leu-14 → Gln	*phoA73*
2	16 (13–16)	20	Leu-8 → Gln	*phoA68*
3	10 (8–10)	2	Δ(Leu-8 Ala-9)	*phoA82*
4	3	1	Leu-10 → His	*phoA93*
5	2 (2–2½)	8	Ala-9 → Glu	*phoA21*
6	1 (1–1½)	14	Leu-14 → Arg	*phoA61*

[a] AP enzyme levels in the mutants are shown as the percentage of wild-type enzyme activity. Percentage levels in parentheses indicate the range of activities seen for mutants of that class. The single number indicates activity of the strain chosen as the prototype for that particular mutant class.

[b] The DNA sequence was determined for one to six independent mutants of each class. In all cases, every member of a single class whose DNA sequence was determined contained the identical nucleotide change. The following mutant alleles were sequenced: class 1, *phoA62*, and *phoA95*; class 2, *phoA24*, *phoA25*, *phoA28*, *phoA50*, *phoA89*, and *phoA68* (sequenced here and also previously [29]); class 3, *phoA29* and *phoA82*; class 4, *phoA93*; class 5, *phoA3*, *phoA21*, and *phoA70*; and class 6, *phoA26*, *phoA628*, and *phoA61* (sequenced here and previously [29]).

[c] The allele designation for a particular class is derived from the last two digits of the prototype mutant strain number (Table 1) for that class.

create a nonsense codon near the fusion joint and potentiate synthesis of a restart β-galactosidase product (28) or deletions resulting in production of a shortened hybrid protein devoid of export signals (36) would also be in this class. Preliminary results indicate that many of the lesions may fall into this latter category, since phage from 2 of the 27 nontransferable Lac$^+$ mutations were analyzed by restriction mapping, and both were found to contain large deletions in the gene fusion (data not shown). We concluded that the nontransferable group of mutations was not likely to readily provide mutants useful for the study of secretion.

Only those 47 mutations that could be successfully transferred to the chromosomal *phoA* gene were studied further. All subsequent experiments with these mutations were performed with a cured nonlysogenic derivative in which the original mutation had been recombined from λ *phoA-lacZ456* to the *phoA* gene on the chromosome. The mutant alleles were assigned a *phoA* designation (i.e., *phoA21*).

Quantitation of export defect by AP enzyme assay. Because the mutations being analyzed here were selected on the basis of their ability to cause internalization of a hybrid protein, we anticipated that when incorporated into an otherwise wild-type *phoA* gene they would interfere with AP export. Immunoprecipitation experiments with a subset of the mutants confirmed that export was impaired. In the mutants a major portion of the newly synthesized AP was not processed to the mature form but instead remained as the precursor species (see below).

We had shown previously for two *phoA* signal sequence mutants that AP enzymatic activity is a reliable assay for AP export. In those mutants, we observed that cytoplasmic precursor was inactive and that the low level of residual AP activity exhibited by those strains reflected the small amount of normal mature AP detectable in the periplasm (29). Thus, enzymatic activity can be used to quantitate the extent of secretion in strains bearing export mutations in the *phoA* gene. We performed enzyme assays on our collection of 47 mutants and were surprised to find that the mutants did not exhibit a continuous spectrum of enzyme activities, but instead fell into six discrete classes (Table 2).

Class 1 mutations appear to cause a modest block to export since strains in this category exhibited a relatively high level of residual AP activity (approximately 30% that of wild type). Class 2 and 3 mutations cause a greater inhibition of export as evidenced by the lower residual activity in these strains (16 and 10%, respectively). Classes 4, 5, and 6 are very severely impaired for export; they exhibited extremely low levels of activity (3, 2, and 1%, respectively). It should be noted that the enzyme assay employed is sufficiently sensitive and accurate to distinguish activities in this range. That classes 4, 5, and 6 are indeed different from one another is further substantiated by a qualitative but sensitive plate assay. On medium containing appropriate concentrations of P_i and XP, a chromogenic substrate for AP, mutants in these three classes are clearly distinguishable by the intensity of blue color in the colonies they form. Under the conditions employed, this plate test only permits phenotypic discrimination within a low range of enzyme activity (less than 10% of the wild-type level).

We considered the possibility that mutants falling within a single class might all contain the same mutation. To examine this issue, we took advantage of a suppressor mutant we had isolated (S. Michaelis, P. Benfey, S. Henry, and J. Beckwith, unpublished data) that specifically suppresses the *phoA61* mutation which had been previously characterized and falls into class 6 as defined here. We believe this suppressor to be a missense suppressor. It causes allele-specific suppression of the PhoA$^-$ phenotype of *phoA61* and maps in or near a tRNA cluster at minute 15 on the *E. coli* chromosome. Furthermore, it displays sensitivity to rich growth medium, a phenotype characteristic of at least one well-studied missense suppressor (15). When AP activity was measured in the presence of this putative missense suppressor, all five of the class 6 mutants tested, and mutants in no other class, could be suppressed, suggesting that all class 6 mutants are identical and are independent isolates of the previously characterized *phoA61* allele. DNA sequence analysis (see below) for two of these suppressible mutations confirmed that they are identical with *phoA61*.

Transferring mutations from the *E. coli* chromosome onto the RF of a single-stranded phage by recombination in vivo. To perform DNA sequence analysis of our *phoA* mutations by the dideoxy sequencing procedure, it was first necessary to move the mutations onto a single-stranded bacteriophage vector. We wanted to avoid the tedious manipulations required to do this by in vitro recombinant DNA techniques. For this reason we took advantage of in vivo homologous recombination to transfer mutations from the bacterial chromosome directly to the double-stranded RF of a single-stranded phage.

This procedure relies on having an M13-type phage suitable as a participant in homologous recombination between sequences in or adjacent to the *phoA* gene. We constructed such a phage, f1 SM103, which carries a partially deleted *phoA* gene and intact flanking sequences. The particular deletion we incorporated into the *phoA* gene on the phage removes approximately half of the DNA encoding the AP protein including its signal sequence (28). f1 SM103 forms white plaques on a Δ*phoA* indicator strain in the presence of XP. The *phoA* gene can be reconstituted by homologous recombination between the chromosome and the phage RF DNA when f1 SM103 is propagated on a *phoA*$^+$ host strain. In this case, blue plaques are detected at a frequency of 10^{-4} to 10^{-5}. The length of homologous sequences available to participate in the exchange between chromosomal and phage DNA greatly influences the frequency of recombinants observed. To promote efficient recombination, we found that 1,200 base pairs of homology on either side of the phageborne deletion was sufficient. Using a different phage,

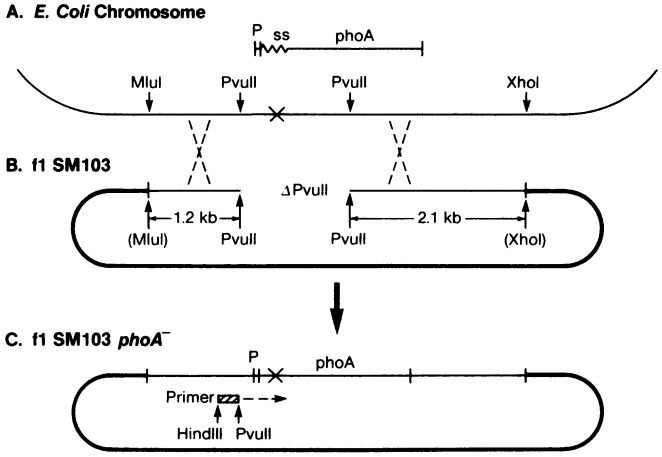

FIG. 2. Strategy for recombining mutations from the chromosome onto a single-stranded phage RF for DNA sequence analysis. The position of the *phoA* promoter (P), signal sequence (ss), and protein-coding region relative to various restriction sites is indicated. In panel A the chromosomal *phoA* gene bearing an export mutation (×) is shown. Two crossover events (dashed lines) between chromosomal and f1 SM103 DNA, on both sides of the *Pvu*II-*Pvu*II deletion (B), will reconstitute on the phage an intact *phoA* gene bearing the *phoA21* mutation as shown in panel C. Thick lines represent vector DNA. *Mlu*I and *Xho*I sites define the boundary between vector DNA and insert sequences (light lines). These sites are noted in parentheses because they were both inactivated during construction of f1 SM102, the parent of f1 SM103. The DNA sequences lying between the (*Mlu*I) and *Pvu*II sites and between the *Pvu*II and (*Xho*I) sites provide sequences homologous to the bacterial chromosome and thus are targets for recombination. The lengths of those homologous sequences are indicated. The *Hin*dIII-*Pvu*II restriction fragment used as a primer for DNA sequence analysis is shown. kb, Kilobases.

f1 *phoA* Δ*Pvu*II (29), which has much less homology available on one side of the deletion (180 base pairs instead of 1,200) we found that the frequency of *phoA*$^+$ recombinants was so low (less than 10^{-6}) that it was impractical to perform routine transfer of mutations by homologous recombination.

We were able to apply this system for moving signal sequence mutations to f1 SM103. Because the color indicator XP is so sensitive that mutants making as little as 10% of the normal level of activity are indistinguishable from wild type, mutations in classes 1, 2, and 3 could be transferred just as described for wild type. On the other hand, mutants in classes 4, 5, and 6 produce very low levels of AP activity. We found, however, that if the indicator strain used for visualizing plaques contained the signal sequence-specific suppressor mutation, *prlA* (10), then blue plaques could be obtained. The incidental finding that the *prlA* mutation suppresses our *phoA* mutations suggests that all our lesions reside in the *phoA* signal sequence. Using the recombination scheme, 15 mutations were transferred to f1 SM103. For classes represented by only one or two mutations (i.e., classes 1, 3, and 4), each mutation was transferred. For classes 2, 5, and 6, f1 SM103 derivatives were obtained for 5 of 20, 3 of 8, and 2 of 14 mutations, respectively.

DNA sequence analysis of mutations which result in impaired export of AP. The nucleotide sequence of the signal sequence coding region was determined for each of the mutations transferred to f1 SM103. This was performed by the dideoxy sequencing technique, using a denatured restriction enzyme fragment lying near the beginning of the *phoA* gene as a primer for elongation (Fig. 2). Six distinct nucleotide alterations were represented among the 17 mutants sequenced (Fig. 3). Each of these six different sequence changes corresponds to a single class as defined by AP enzyme activity (Table 2). The lesions in the two *phoA* signal sequence mutations previously reported, *phoA68* and *phoA61*, were identical to those in the two most frequent classes studied here, 2 and 6, respectively. It is noteworthy that all the mutations we found were due to alterations in the signal sequence and not elsewhere in the protein, suggesting that the integrity of the signal peptide is necessary for secretion, while the conformation of other regions of the protein is not as crucial for facilitating export.

Five of the lesions we found are missense mutations resulting from transversion, and one is a deletion of six base pairs. They all affect residues in the hydrophobic core of the signal sequence. Since the relative strength of these mutations in terms of interfering with export can be quantitated by AP activity, our data indicate that the strongest block to export results when a charged amino acid is introduced into the signal sequence, while substitution of a polar residue or simply deletion of two residues results in a less extreme export defect (Table 2).

Kinetics of AP export in *phoA* signal sequence mutants.

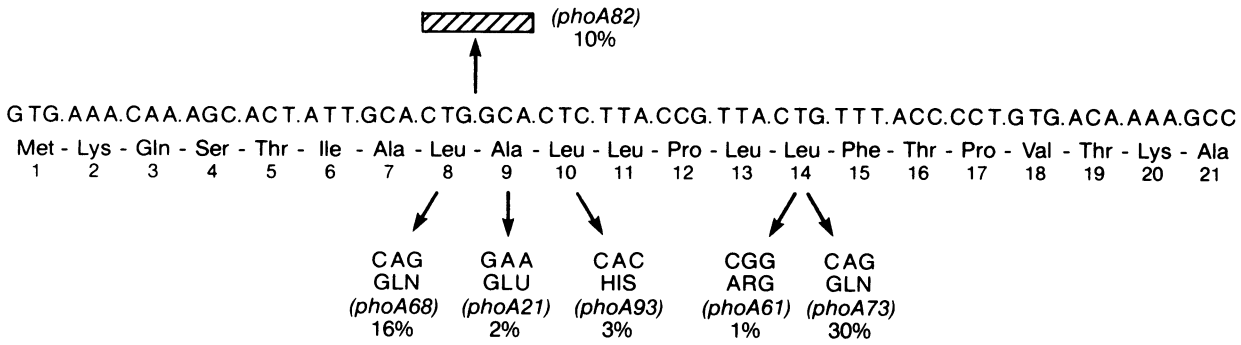

FIG. 3. DNA nucleotide sequence changes leading to amino acid alterations in the *phoA* signal sequence. The wild-type *phoA* signal sequence coding region with its corresponding amino acids is shown. The small number under each codon designates the position of that residue in the AP precursor. Mutations with their resulting amino acid alterations are indicated by arrows. The two codons deleted by the *phoA82* allele are indicated by a bar. The allele number used to designate each mutation is given in parentheses. Percentage indicates the amount of residual AP enzyme activity in a strain bearing the mutation; the lower the percentage of activity, the more severe is the export block caused by that mutation.

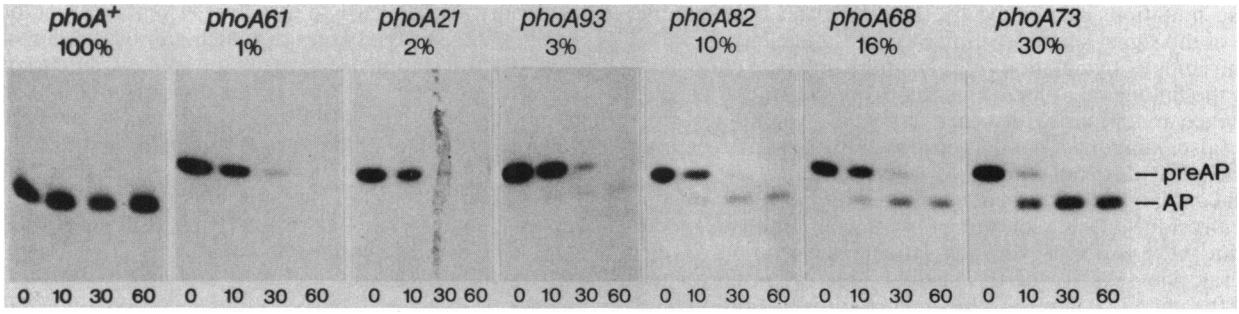

FIG. 4. Pulse-chase analysis to determine kinetics of AP export in mutants. Cells were pulse-labeled with [^{35}S]methionine for 30 s, and radioactivity was chased for various lengths of time. Samples were immunoprecipitated and electrophoresed as described in the text. The positions of mature and precursor AP are indicated. The percentage indicated above each panel refers to the amount of residual AP enzyme activity present in that mutant. The number shown below each lane indicates the time of chase (minutes), the zero time point thus representing a pulse.

Defective export of AP can conveniently be detected in a pulse-chase experiment. Cells are first radiolabeled, and then AP is immunoprecipitated and electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel such that the precursor and mature species are resolved. Since processing is believed to occur only after a protein is translocated through the membrane (21), the ratio of the amount of the processed form to that of the precursor species at any particular time is a measure of how much secretion has occurred during the period after incorporation of radioactivity. A large amount of the mature product relative to precursor indicates efficient secretion, whereas the presence of a high proportion of precursor indicates poor secretion. For wild-type AP, after a 1-min pulse-label (with no chase period), all of the radiolabeled product was in the mature form; no precursor could be detected (Fig. 4). This rapid and efficient removal of the signal peptide from nascent AP molecules indicates that translocation of AP across the cytoplasmic membrane occurs either cotranslationally or in an extremely rapid posttranslational manner.

We examined the processing kinetics of one representative for each of our six classes of AP signal sequence mutants (Fig. 4). We found that the temporal processing profile of the mutants differs strikingly from that of wild type. After a 1-min pulse-labeling period with no chase, only the precursor is detected for the mutants, which contrasts with the wild type in which only the mature species is present (Fig. 4, left lane of each panel). Therefore, the signal sequence mutants appear to interfere with the operation of the normally rapid protein export pathway for AP in *E. coli*.

The fate of the pulse-labeled precursor found in the mutants was followed during a chase period. This analysis revealed that the block in export for newly synthesized AP molecules is not permanent or absolute, since for all of the mutants, some amount of mature AP product did eventually appear during the chase period with delayed kinetics (Fig. 4). This observation suggests that a slow, posttranslational mechanism can promote secretion. The efficiency of this export process varies considerably depending upon the signal sequence allele, so that, as expected, mutants that produce greater levels of AP enzyme activity eventually convert a greater fraction of the total amount of labeled product to the mature form.

It should also be noted that in all the signal sequence mutants the major fraction of the AP labeled during the pulse is degraded during the chase period; the apparent half-life of this degradative process is approximately 5 to 10 min (as determined by densitometry scanning of the autoradiograms in Fig. 4).

Thus, two competing processes, degradation and secretion, contribute to determining the fate of the precursor. For wild-type AP, secretion is so rapid that the contribution of degradation is insignificant. For the signal sequence mutants, however, secretion is considerably slower so that degradation dominates. In any mutant, the effectiveness with which secretion competes with degradation is indicated by the final fraction of AP that is converted to the mature form and reflects the rate constant of secretion for that mutant.

DISCUSSION

To obtain mutants defective in secretion, we used a genetic selection which demands that an AP–β-galactosidase hybrid protein be shifted in its location from the membrane to the cytoplasm. Since the membrane location of the hybrid protein requires the presence of a functional AP signal sequence at its amino terminus, the selection yields primarily mutants which contain lesions in the *phoA* signal sequence. In addition, certain other mutants obtained in this selection contain unlinked mutations which may affect components of the secretory apparatus of the cell. One of these mutants, which is cold sensitive for growth, will be described in detail in a subsequent publication (Gardel et al., in preparation).

In this study we analyzed 47 mutants which contain alterations of the AP signal sequence. Each of these mutants exhibits some residual AP enzyme activity. We have proposed previously that the level of enzyme activity in these strains is a direct measure of the amount of AP exported to the periplasm (29). The evidence presented here and elsewhere (16, 26a) supports the conclusion that AP is enzymatically active only when it is exported through the cytoplasmic membrane; the internalized protein is inactive and degraded. For instance, it can be seen from a comparison of Table 2 and Fig. 4 that the amount of enzymatic activity measured for each signal sequence mutant corresponds to the amount of mature AP seen on gels after pulse-chase immunoprecipitation experiments.

On the basis of their levels of AP activity, the signal sequence mutants reported here fall into six classes. DNA sequence analysis was carried out on representatives of each class. These studies revealed that, of those analyzed, all mutants in each class contained the same genetic alteration.

These mutations all reduce the length of the hydrophobic core of the signal sequence either by introducing hydrophilic amino acids or by deletion (Fig. 3). Our results are consistent with the findings that have emerged from the study of signal sequence mutations with other exported proteins (3), and they also support a model proposed to describe certain important features of the signal sequence (2).

The mutations described here alter amino acids over a seven-residue stretch of the *phoA* signal sequence, from leucine at position 8 through leucine at position 14. In general, the severity of the secretion defect is correlated with the chemical nature of the amino acid substitution, and supports the notion that the general hydrophobic character of the signal sequence, rather than its specific steriochemical structure, may be important for secretion. Insertion of a full positive (arginine) or negative (glutamic acid) charge toward the middle of the hydrophobic core almost completely inhibits secretion (less than 2% for *phoA61* and *phoA21*). Interestingly, insertion of a histidine residue in this region also produces a severe secretion defect (only 3% for *phoA93*). Thus, it appears that at least with regard to the interactions involving the signal sequence, histidine (whose pK_a is close to physiological pH) behaves as a highly charged amino acid. Insertion of a polar but noncharged residue (glutamine) leaves a substantially more functional signal sequence (16 and 30% for *phoA68* and *phoA73*, respectively), whereas a two-residue deletion in the hydrophobic core of the signal peptide results in an intermediate level of activity (10% for *phoA68*). It is also noteworthy that an identical leucine-to-glutamine substitution at different positions in the AP signal sequence results in very different levels of export competence: the leucine-to-glutamine change at position 14 in *phoA73* allows almost twice as much AP export as exactly the same change at position 8 in *phoA68* (30 versus 16%, respectively).

While we did not determine the nucleotide sequence of all 47 mutations obtained here, it appears from the ones examined that, beyond the six specific alterations seen, no others were picked up in this selection at comparable frequencies. One explanation for these results is that other alterations do not have serious effects on signal sequence function. Emr and Silhavy (11) have proposed that, in the case of the LamB signal sequence, only certain of the amino acids in the hydrophobic domain are important for the export process. However, it seems possible from our data that the absence of other mutations from our collection is simply a matter of unequal mutability of sites. From Table 2, it can be seen that 42 of 47 mutants analyzed fell into only three classes (2, 5, and 6). If we assume that most or all of these mutants represent the same nucleotide sequence alterations, then it would appear that these sites are particularly mutable. For future analysis, it would be of interest to examine other lesions, e.g., at positions 11, 12, and 13, to determine whether the absence of such mutations from our collection is a question of mutability or is due to the less crucial role of these residues for signal sequence function. Such mutants could be generated by in vitro techniques. We also point out that the deletion represented by *phoA82*, which was isolated twice, removes a region bounded by the 5-base-pair repeat GCACT. Miller has shown previously that most deletions could be explained by unequal crossingover between short homologous DNA sequences (31).

Our analysis of the kinetics of AP export in pulse-chase experiments (monitoring processing of the precursor species to mature AP) demonstrates that the *phoA* signal sequence mutations prevent normal operation of the *E. coli* export machinery with regard to AP. In the mutants, mature AP appears with delayed kinetics which can be explained by a slow posttranslational export of the mutant precursor protein. The efficiency of this posttranslational process for each mutant can be quantitated by enzyme assay or estimated from the amount of mature product seen in an autoradiogram and reflects the rate constant for secretion in that mutant.

Protein translocation into the endoplasmic reticulum of eucaryotic cells is thought to be obligatorily cotranslational (4, 5). The data of Randall and co-workers (21, 34), Wickner and co-workers (9), and Koshland and Botstein (22), as well as the results from in vitro protein secretion studies (8, 32, 40), suggest that such coupling is not required for protein export in *E. coli*. Our observations here with AP signal sequence mutants support the contention that secretion can occur via a posttranslational route in *E. coli*. In a number of other instances in which normal secretion is impaired, posttranslational secretion has also been detected. Thus, for β-lactamase (23), lipoprotein (39), and maltose-binding protein (35), signal sequence mutants exhibit posttranslational export. In addition, for mutants with a defective secretory apparatus (e.g., *secA* and *secB* mutants) (12, 24) or a blocked apparatus due to a hybrid protein (20), precursors are converted to the mature form in a posttranslational fashion.

The posttranslational export pattern for AP observed here with the signal sequence mutants could involve a fundamentally different mechanism from the normal secretion pathway which is extremely rapid and cotranslational. Alternatively, it could represent operation of the standard secretion apparatus with altered kinetics owing to the interference with steps that are normally signal sequence dependent. Examination of the effect of *sec* or *prl* mutations on the slow export kinetics of signal sequence mutants may help distinguish between these two possibilities.

ACKNOWLEDGMENTS

We thank Jeff Felton for many helpful discussions. We also thank Aaron Mitchell and Jeff Felton for critically reading the manuscript, Hiroshi Inouye for contributing DNA, and Theresia Luna for technical assistance.

This work was supported by American Cancer Society grant MV-5K.

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